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FURTHER STUDIES ON THE METABOLISM OF *PROTEUS VULGARIS*; ITS STABLE L-PHASE
AND THE PLEUROPNEUMONIA-LIKE ORGANISMS

(Following is the translation of an article by O. Kandler,
G. Zehender and J. Mueller, Botanic Institute, University of
Munich, which appeared in the German language periodical
Archives for Microbiology, Vol. 24, pages 209-218 (1956).
Translation performed by Constance L. Lust.)

In continuing our studies on metabolism of L-phases and PPLO we performed experiments concerning substrate utilization and the sensitivity of poisons on these organisms. Previous studies (Kandler et al 1956) showed that the oxidative metabolisms with respect to syntheses had about the same extent in stable L-phases and in PPLO. The present trials were done to show whether qualitative differences exist in metabolism in three forms of life which may be manifested in an altered substrate requirement or in a different sensitivity to metabolic poisons. These studies were designed as preliminary experiments for a thorough, detailed enzyme-chemical investigation.

Materials and Methods

The same strains were used as in the earlier experiments (Kandler and Kandler 1955). The nutrient broth was the same also. In liquid cultures the phosphate concentration was quadrupled. Contrary to earlier experience the stable L-phase of *P. vulgaris* grew very well in this nutrient solution. It may be due to a slow adaptation to this nutrient medium over a series of passages (150 up to now). The continuing cultures for transfer were always grown on solid medium, while the material for the measurements of metabolism were grown in 300 ml liquid medium. Incubations were at 37°C. Inoculation was done by streaking or merely throwing a piece of agar into the medium. Sometimes liquid to liquid transfers were made, but here the danger of contamination is greatly enhanced.

Harvest of the organisms came soon after the main growth (log phase), for *P. vulgaris* after 26 hours; for the L-phase and PPLO after 3-4 days. The organisms were centrifuged for 10 minutes at 15,000 x g, washed with distilled water and then used for measurements. For all three organisms it was established in preliminary work that a buffer concentration of M/30 to M/45 and a pH of 7.8-8.2 was optimal. The organisms were stable for longer periods in these phosphate buffers. This was checked by giving substrate after 1-8 hours to equal suspensions. Up to 4 hours the respiration after substrate addition was equal, and only after longer times of starvation did the respiration rate decrease in L-phase and PPLO. In *P. vulgaris* the starvation period could be extended much more.

The effect of poisons on growth was tested by preparing the nutrient medium with inhibitor and then inoculation with organisms. Onto each plate 3 PPLO strains and L-phase were inoculated, so that identical conditions existed. *P. vulgaris* was inoculated onto its own plate. After

12 to 24 hours the results were read and relative growth was expressed. If no macroscopic growth was visible the stereomicroscope was used to insure that no small colonies had formed.

All respiratory measurements were done using the manometric method of Dixon (1953). Total N was determined in the suspension by the Kjeldahl method (Klein 1932). The substrates were adjusted to pH 7.8 with sodium hydroxide; the acids then were present as sodium salts.

Results

1. Experiments on the oxidative degradation of various substrates.

Previously (Kandler and Kandler 1955) we showed that the L-phase and the bacteria are not different in their capacity to form acid from various carbohydrates. In this report the qualitative bacteriological method of carbohydrate substrate utilization which was susceptible to several errors was replaced by the manometric measurement of oxygen uptake after addition of a particular substrate.

It is well known that mutants appear frequently in bacteria and these mutants may have lost (or have altered) the ability to utilize substrates. It is entirely possible that during the deep-rooted transition of the L-transformation such alterations can occur in greater numbers. Therefore we determined the velocity of oxygen uptake after addition of the physiological most important carbohydrates, organic acids, amino acids and alcohols.

From one preparation to the next the absolute value of N^QO_2 (oxygen consumption per hour and mg total N in mm^3) was different even for the same substrate. This may be because age and culture conditions are not always completely identical. In order to have a standard for comparison, a control sample of peptone was run; this was considered the most advantageous substrate. The respiration of other substrates was then expressed in terms of percent of "peptone-respiration". From a single suspension as many as possible tests for substrates were done (usually 12) so that the relative values for each trial were constant.

All substrates were used in concentration range from 0.01 to 0.1 M. The values listed in table 1 are maximum values; particularly with organic acids much lower values for respiration maybe obtained, compared to peptone. Here we do not intend to discuss the details of the factors that influence turn over rate of substrates.

As may be seen in table 1, no qualitative difference in respect to ability to oxidize various substrates exists between bacteria-form and L-phase. A clear quantitative difference exists for the lower, organic acids, which compared to other substrates are utilized better by L-phase than by bacteria. However, the absolute velocity of respiration is considerably lower in the L-phase. The N^QO_2 of the peptone respiration varied between 90-150 for L-phase, while for *P. vulgaris* it was 600-1200. With optimal substrate and during log phase of growth the values were still higher (Kandler et al 1956).

Substrat	<i>Protrus vulgaris</i> %	L-Phase %	Substrat	<i>Protrus vulgaris</i> %	L-Phase %
Saccharose	45	40	Asparaginsäure	60	50
Glucose	45	50	Asparagin	40	50
Dextrin	2	10	Glutaminsäure	30	24
Stärke	2	5	Leucin	25	30
Maltose	39	40	Methionin	25	25
Fructose	30	35	Tyrosin	20	20
Lactose	2	6	Alanin	10	10
Galaktose	45	35	Glykokoll	5	7
Ribose	15	20			
Essigsäure	80	100	Methanol	2	3
Ameisensäure	50	75	Äthanol	5	5
Bernsteinsäure	70	110	Butanol	2	3
Milchsäure	80	130	Glycerin	30	23
Brenztraubensäure	30	14			
Äpfelsäure	25	12	Leeratmung		
Glykolsäure	15	12	ohne Substrat	2-3	3-5
Propionsäure	2	5			
Malonsäure	2	5			
Weinsäure	2	5			

Table 1 - Oxygen utilization in % of "peptone respiration", after adding various substrates pH 7.8, 27°C.

Similar experiments with PPLO strains gave a totally different result (mainly strain "Findlay" was used). As reported earlier (1955) only when a combination of yeast extract, peptone and glucose is added do PPLO take up oxygen actively. This is not just because of an additive effect of the addition of the individual substrate. Without adding other substrates only glucose, maltose, fructose and gallactose effected a measurable rate of respiration in the first hour. This decreased again rapidly. Mannose, Sorbose, Phamnose, arabinosl and xylose were without effect. Also organic acids, amino acids and alcohols did not lead to much stimulation over blanks. Combined with yeast extract the respiratory effect of carbohydrates, eg. glucose, starch, galactose, fructose, cellboboise (in order of relative effect) was slighter greater. The other substrates were still without effect.

Vitamins, which are known to be coenzymes for respiration, were added to measure glucose utilization. Only ascorbic acid stimulated oxygen consumption. However, this was because it was itself oxidized (not mere glucose). Vitamin B₁, B₆, pantothenic acid, folic acid, biotin, etc showed no clear conclusions.

The fact that our PPLO strains did not oxidize organic acids was surprising. Practically all bacteria attack these substrates rapidly.

As the earlier trials with L-phase showed, this ability to oxidize acids is not lost in the L-transformation. If, as some authors assume (Dienes and Weinberger 1951, Tulasne, 1951, 1953) PPLO are only stable

L-phases whose bacteria forms are unknown, then one should search for bacteria that have similar nutritional requirements. It may be that a slow reduction of substrate usage is independent of the actual transformation process. For this there are no points of reference as yet.

2. Trials on the effect of various poisons of respiration.

Various poisons played a major role in the elucidation of metabolic pathways in the last ten years. By using poisons with known, specific effects, we can now say whether the metabolic pattern of an organism proceeds via known mechanisms, or if other paths must be called forth.

We tested whether *P. vulgaris*, L-phase and PPLO were inhibited to the same degree by poisons, or if one of these organisms shows other characteristics. In figure 1 an abbreviated schema for carbohydrate degradation is presented and the probable locus of action of some poisons (see James 1953). Moniodoacetic acid and fluoride act on the Embden-Meyerhof pathway; arsenite blocks lipoic acid and thru the transfer of pyruvic acid into the citric acid cycle; KCN and azide inactivate iron in cytochrome a-oxidase. Azide also uncouples phosphorylation as does 2,4 DNP. Arsenate is used as a universal phosphorylation poison.

The effectiveness of the poisons was determined in two experiments. First nutrient medium contained various poisons and growth was watched. These results are presented in table 2. Second, manometric trials were done where liquid cultures of organisms were first grown on optimal substrate, and then various poisons were added in solution. The O_2 used after an hour (after the poison had acted fully) is presented as percent of the unpoisoned culture (table 3). (The action of the poisons came at different times.)

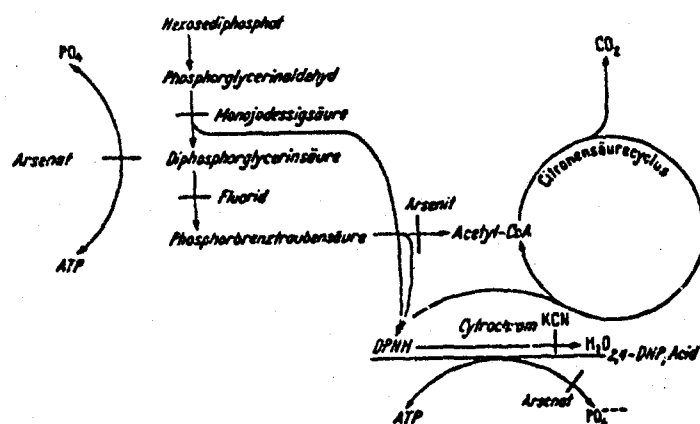


Figure 1 - Schema for carbohydrate degradation with places where poisons act indicated

Gift	Konzentration	PPLO	L-Phase	<i>Proteus vulgaris</i>
KCN	m/100	++	-	++
	m/1000	++	+	++
2,4-Dinitrophenol	m/500	++	-	++
	m/5000	++	++	++
Azid	m/10	+	-	-
	m/100	++	+-	+-
	m/1000	++	++	++
Arsenat	m/10	+	-	-
	m/100	++	-	-
	m/1000	++	+	+0
	m/2000	++	++	++
Arsenit	m/100	+	-	-
	m/500	++	-	+-
	m/1000	++	+-	+
	m/10 000	++	++	++
Monojodessigsäure	m/200	-	-	+-
	m/500	-	-	+
	m/1000	-	-	++
	m/10 000	-	+	++
	m/20 000	-	++	++
	m/50 000	+	++	++
	m/100 000	++	++	++
Fluorid	m/10	-	+	++
	m/50	-	++	++
	m/100	-	++	++
	m/200	+	++	++
	m/500	++	++	++

Table 2 - Growth on solid medium in presence of various poisons
- none, +- weak, + good medium, ++ very good, +0 growth
without colony formation

The comparison of both table 2 and 3 shows good agreement. The measurement of growth is of course much more accurate than respiration. It is sensitive at lower concentration. Only quantitative differences exist between bacteria and L-phase; in the sense that L-phase is always more sensitive in the growth test. A clear difference in the inhibition was seen with iodoacetic acid. It was markedly more effective against L-phase than against bacteria. Arsenate and azide both have a diminished effect on L-phase. If one considers that these values may vary in different batches by $\pm 10\%$ than it is basically demonstrative of good agreement. In any case it does not support that a different mechanism of respiration is present in L-phase than in the bacterial form.

On the other hand, the PPLO show a completely different behavior (8 strains of PPLO were studied). In both test they are very sensitive to iodoacetic acid and fluoride. All other poisons do not inhibit even in rather high concentrations. KCN even stimulates respiration considerably. These effects are reproducible and have been repeated in other organisms. However, an explanation is not at hand at this time. It may be that heavy metals, which inhibit metabolism are complicated by KCN, but this explanation probably is not valid at high concentrations.

Sub	Concentration	PPLO	L-Phase	<i>Proteus vulgaris</i>
KCN	m/1000	150	35	62
	m/10 000	150	60	85
2,4-Dinitrophenol	m/50	100	20	48
	m/150	100	38	66
Azid	m/10	100	30	6
	m/100	100	75	18
	m/1000	100	85	70
Arsenat	m/10	100	55	—
	m/100	100	94	18
	m/1000	100	75	25
Arsenit	m/80	65	—	0
	m/500	75	33	26
	m/5000	100	60	—
Monojodosaigsäure	m/500	—	25	40
	m/1000	—	32	75
	m/5000	11	48	90
	m/10 000	22	70	—
Fluorid	m/10	30	60	80
	m/50	50	100	100

Table 3 - Oxygen utilization in % of uninhibited controls

Of particular interest was the relative resistance to arsenate. These studies were done on medium that had no phosphate added, because this would overcome the arsenate effect. The media were not completely phosphate-free, since peptone contains large amounts. This latter amounts of phosphate do not suffice to prevent the arsenate inhibition in other organisms, eg. *Proteus vulgaris*.

The behavior of PPLO in respect to the metabolic poisons leads one to conclude that they are not dependent on the cytochrome system nor on the citric acid cycle in their aerobic metabolism. Only the arsenite sensitivity speaks for an effect in oxidation of organic acids. It appears that the usual phosphorylation mechanism is either missing or very much reduced, because otherwise the resistance to arsenate, DNP and azide is not understandable. The Embden-Meyerhof pathway appears to play a major role, since both iodacetate and fluoride are highly effective in their inhibition.

Discussion

The studies about substrate utilization and poison sensitivity imply that in the L-phase transmission no qualitative change occurs in metabolism. The L-phases in all probability use the same enzymes as do the original bacteria. A slow change in continuous passages of L-phase has not been detected yet after 150 passages.

The PPLO-strains, however, in all certainty use another degradative pathway, especially on other terminal oxidase system. They are characterized and differentiated from bacteria due to their ability of oxidatively use organic acids and amino acids, and their resistance against a series of metabolic poisons. We studied *E. coli*, *Pseudomonas* types, *Flavobacteria*

spec., *Achromobacter*, *Micrococcus*, *Sarcina lutea*, *B. subtilis* and mesentericus, *Bact. linens* and *Caulobacter vibrioides* against poisons, and none behaved similar to PPLO. *Sarcina* was especially resistant to arsenate, which was similar to PPLO. But *Sarcina* was sensitive to azide and arsenite; PPLO was not.

The identity of metabolism of L-phases and bacteria, and the special phase of PPLO appears to be an important point in reference to the relationship between L-phases and PPLO. We believe from these data, and previous data (Kandler and Kandler 1955, 1956) that PPLO represent a distinct class of organisms, and they are not stable L-phases. At least not very recently derived L-phases. It is possible that PPLO were related phylogenetically to bacteria long ago, but then there is still no need to designate them L-phases. They represent an individual systematic unit as did other phylogenetic groups of organisms.

Studies of metabolism of L-phase have yielded little information about their origin. PPLO, however, should be studied in enzymological detail with respect to their metabolism.

Summary

By using manometric respiration measurements it was determined that the stable L-phase of *P. vulgaris* utilized the same substrates as does the bacteria from which it was isolated. The sensitivity to various poisons is also the same (with some minor deviations).

Eight strains of PPLO oxidized only a few carbohydrates and then minimally; no organic acids, amino acids and alcohols. They are resistant to high doses of KCN, DNP, azide, arsenite and arsenate. Implications are that they possess other enzyme systems for substrate degradation than do most other organisms. Because of high sensitivity to iodoacetate and fluoride it may be assumed that degradation is via the Embden-Meyerhof pathway to pyruvic acid.

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